AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2018 – 2020

TITLE: RNA Seq-guided identification of genes for production of a novel antimicrobial by the biocontrol agent *Pseudomonas fluorescens* A506

RESEARCH LEADER: Dr. Virginia Stockwell, USDA-ARS Horticultural Crops Research Unit, Corvallis, Oregon and Department of Botany and Plant Pathology, Oregon State University (Courtesy Appointment)

CO-RESEARCH LEADER: Dr. Qing Yan, Department of Botany and Plant Pathology, Oregon State University; currently Assistant Professor, Dept. of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT.

EXECUTIVE SUMMARY:
Fire blight is a destructive bacterial disease of pear and apple flowers and shoots caused by *Erwinia amylovora*. The commercial biocontrol agent *Pseudomonas fluorescens* strain A506 (A506) reduces disease by competitive exclusion of the pathogen on flowers. We discovered that A506 produces an unknown antimicrobial compound in iron-amended media that is lethal to *E. amylovora*. The overarching goal of the research project was to identify the antibiotic produced by A506. Previously, we attempted to isolate the antibiotic, but were unsuccessful. We know that the antimicrobial is a polar compound that passes through small-pored dialysis membranes. Previously, we sequenced the genome of A506, however genomic analysis using the program to identify secondary metabolites based on sequence (antiSMASH) did not yield clues as to the genes involved in production of the antimicrobial. Production of the compound is under the *gacA-gacS* regulon because a *gacS* mutant of A506 did not produce the antimicrobial. A506 carries a native plasmid (pA506). Deletion of the plasmid did not impact production of the antimicrobial, leading us to the conclusion that the biosynthetic operon is located on the chromosome of A506.

We used RNA-Seq to identify genes and gene clusters are differentially expressed in A506 cultured in iron-poor and iron-rich media and the *gacS* mutant of A506. We hypothesized that genes and clusters upregulated when iron is abundant and downregulated in the *gacS* mutant may be associated with the biosynthetic genes for antimicrobial production. Knowing which genes or clusters encode for the antimicrobial compound can guide isolation and identification.

From transcriptomics, we identified several candidate gene clusters that were upregulated in iron rich media and downregulated in the *gacS* mutant. We used a multi-step deletion-mutagenesis method to remove single genes or clusters (several adjacent genes) from A506. The mutants were tested for antimicrobial production in a culture-based assay. Among the mutants tested, deletion of two clusters, separately, abolished production of the antimicrobial. Interestingly, when we removed those clusters from a plasmidless derivative of A506, the antimicrobial compound was produced. We concluded that the deleted clusters were

not directly involved in biosynthesis of the antimicrobial. We speculate that the products of the clusters may have influenced expression of a regulatory gene on the plasmid. Moving forward, we will continue to delete additional genes and test those mutants for antimicrobial production. Our long term goals are to identify the antimicrobial, determine its properties and potential for control of bacterial diseases.

OBJECTIVES:

1. Identify genes that are associated with production of an unknown antimicrobial in A506.
2. Build a GFP-based reporter system for the genes identified in Objective 1.
3. Increase production of the antimicrobial compound for characterization.

PROCEDURES: We focused our research efforts on Objective 1. Identifying genes that are involved in production of the unknown antibiotic of A506.

From transcriptomics research on differential gene expression by A506 and a *gacS* mutant of A506 cultured in iron-amended media, we identified groups of genes that may be involved in the biosynthesis of the unknown antimicrobial. We removed specific genes or gene clusters from the A506, and then tested the mutants for antimicrobial production.

The following (Figure 1) is a schematic of the multi-step process that we used to precisely remove a gene or genes of interest without disturbing the function of neighboring genes. A benefit of the method that we used is that it does not introduce additional markers or abnormal proteins in a generated mutant, thus provides solid evidence to understand the gene's function. More importantly, because of the clean background of the deletion mutant, mutation of multiple genes in one single mutant can be readily achieved to test additive and/or synergistic effect of interested genes.

Two DNA fragments (up and down), that flank the target gene, were generated by PCR amplification and cloned into a suicide vector to make a deletion construct. The deletion construct was then transformed into A506, and integrated into bacterial chromosome via homologous recombination. The mutant generated in this step is resistant to kanamycin because of the *Km*^{R} gene, and sensitive to sucrose because of the *sacB* gene, that were integrated into the A506's genome along with the deletion construct. The second homologous recombination event removes both the deletion construct and the target gene, which results in a deletion mutant. The deletion of a target gene in the mutant can be identified by PCR amplification (Figure 2), and the generated mutant is ready for the antibiosis assay (Figure 3). When needed, the process was repeated in a plasmidless derivative of A506 for further testing.

Figure 1. Diagram of deletion mutagenesis process to remove a gene of interest as described above. Two DNA fragments (labeled up and down) flanking a gene of interest were cloned into a suicide vector to make a deletion construct. The deletion construct is introduced into strain A506 and integrated into the chromosome at the location where we want to remove the gene of interest. The gene or genes of interest are deleted after two homologous recombination events. The suicide vector also was lost because it is incompatible with A506 and not maintained.

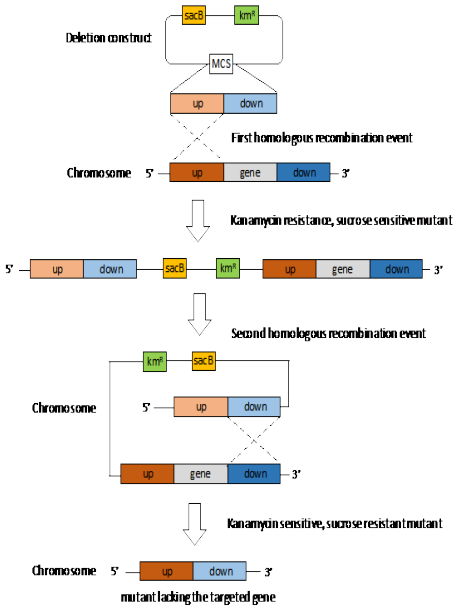


Figure 2. Identification of deletion mutants using PCR amplification. Primers that target the upstream and downstream of the gene cluster of interest were used in PCR amplification. "mutants" and "+" indicate PCR reactions using genomic DNA of putative mutants, and genomic DNA of wildtype A506. "– " indicates the negative control using water only in the PCR reactions. Mutant 1 (in red) was reversed back to wildtype, and mutants 2 to 6 (in green) lack the gene cluster of interest. The PCR product of the mutants is only 1 kb, showing the loss of the 4.1 kb region of the genes of interest.

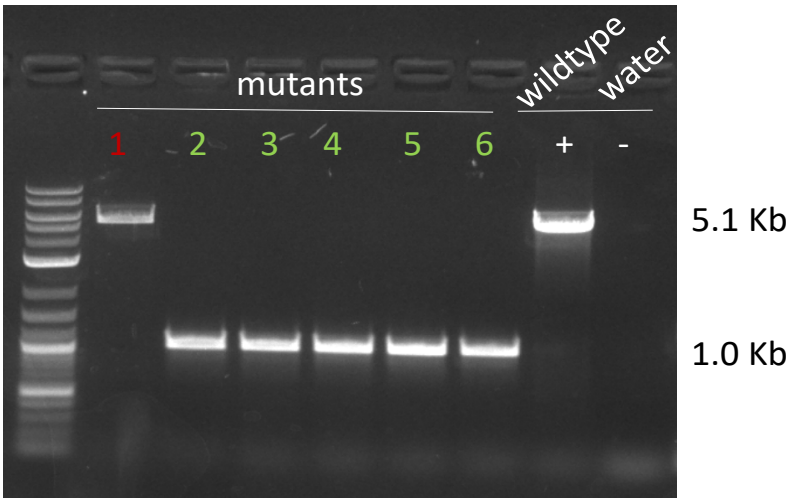
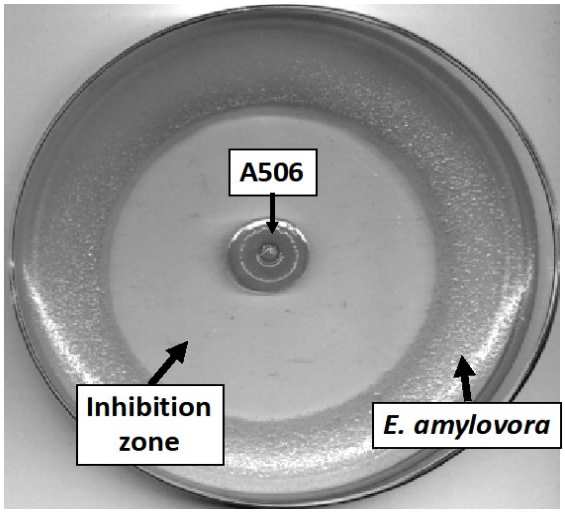


Figure 3. The antibiosis assay is conducted on a defined medium (925 minimal medium) amended with potassium gluconate and 0.1 mM iron citrate. A506 or mutants are cultured on the solidified medium for 48 hours at 27°C. The medium is then overlaid with soft agar containing *Erwinia amylovora*, the fire blight pathogen. Within 48 to 72 hours, inhibition zones (areas where *E. amylovora* cannot grow due to the excretion of the antimicrobial compound into the medium by A506) are visible. The outer edge of the zone of inhibition is marked and measured. We are searching for mutants of A506 that do not produce the antimicrobial and inhibit the growth of *E. amylovora*, which is visualized as no visible zone of inhibition.



SIGNIFICANT ACCOMPLISHMENTS:

1) We analyzed our transcriptomic data sets comparing gene expression by A506 cultured in media with and without iron to look at which genes are expressed when iron is abundant. These are the conditions where we expect the antimicrobial to be produced.
2) We analyzed our transcriptomic data sets comparing gene expression by A506 and a *gacS* mutant of A506. g*acS* is a central regulatory gene for the production of secondary metabolites including antimicrobials; when disrupted, the expression of many genes is altered. The *gacS* mutant of A506 does not produce the antimicrobial. We anticipate that genes for antimicrobial biosynthesis would be downregulated in the *gacS* mutant compared to A506.
3) Comparison of the differential gene expression profiles in iron-amended media and in the *gacS* mutant led us to select between 12 to 20 genes or gene clusters to target as potential biosynthetic regions of the antimicrobial produced by A506.
4) We established procedures to construct and confirm deletion mutants of A506. Additionally, we optimized the culture-based bioassay to detect antibiosis.
5) We tested a five mutants of A506 with deletions or insertions in single genes associated with iron–acquisition. Each of these mutants in the bioassay produced a sharp zone of inhibition and the antimicrobial.
6) We successfully generated mutants of A506 with deletions in genes of interest identified from our transcriptome experiments. Between 3 to 8 colonies were selected from each

mutagenesis experiment, confirmed that the gene(s) of interest was deleted, and then tested for antimicrobial production in the culture-based bioassay.

7) One mutant A506 ΔPflA506_3388 was constructed in a gene cluster that had initially been annotated as a microcin (peptide antibiotics) biosynthetic cluster. The removal of this gene did not affect antibiosis, so we conclude that the the unknown target compound was not encoded by this cluster.
8) Two of the deletion mutants (one with 6 genes deleted and one with four genes deleted) produced either small zones of inhibition in the bioassay or no zone of inhibition. We repeated the mutagenesis procedure for these gene clusters in a A506 derivative that lacks the plasmid pA506. In the repeated bioassay, large zones of inhibition were observed with the mutations constructed in the plasmidless A506. We conclude that the deleted chromosomal genes are not biosynthetic genes for the antimicrobial of A506. We speculate that gene products of these clusters affect putative regulatory gene(s) on the plasmid, resulting in negative results in the bioassays.

BENEFITS & IMPACT:

We recognize that we have not identified the biosynthetic genes for the antimicrobial produced by A506. Nonetheless, through our experiments we are narrowing the list of candidate clusters. We anticipate that the antimicrobial will be novel, as bioinformatic searches and programs such as antiSMASH have not identified known genes or clusters for characterized antimicrobials in the genome of A506.

The regulatory elements influencing antibiosis by A506 are more complex than anticipated. The influence of the plasmid on antibiosis is interesting. The plasmidless strain of A506 produces the iron-regulated antimicrobial, similar to the wild type A506. Yet, some deletions in the chromosome of wild type A506 abolish antibiosis, but antimicrobials are produced when the same mutations are made in plasmidless A506. The differential regulation is intriguing and worthy of additional study.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM: None

FUTURE FUNDING POSSIBILITIES: Future funding will be dependent on the stability, specificity, and type of antimicrobial is produced by A506. Funding possibilities could include programs targeted for applied research in plant disease control or other bacterial diseases, if the antimicrobial can be identified and isolated. Future funding could also include fundamental research on the iron regulon of bacteria, the influence of the plasmid on gene expression, or the biosynthesis and bioinformatics of novel antimicrobials.